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Molecular genetic analysis of *Dichelobacter nodosus* proteases AprV2/B2, AprV5/B5 and BprV/B in clinical material from European sheep flocks

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ABSTRACT

Dichelobacter nodosus, the etiological agent of ovine footrot, exists both as virulent and as benign strains, which differ in virulence mainly due to subtle differences in the three subtilisin-like proteases AprV2, AprV5 and BprV found in virulent, and AprB2, AprB5 and BprB in benign strains of *D. nodosus*. Our objective was a molecular genetic epidemiological analysis of the genes of these proteases by direct sequence analysis from clinical material of sheep from herds with and without history of footrot from 4 different European countries. The data reveal the two proteases known as virulent AprV2 and benign AprB2 to correlate fully to the clinical status of the individuals or the footrot history of the herd. In samples taken from affected herds, the *aprV2* gene was found as a single allele whereas in samples from unaffected herds several alleles with minor modifications of the *aprB2* gene were detected. The different alleles of *aprB2* were related to the herds. The *aprV5 and aprB5* genes were found in the form of several alleles scattered without distinction between affected and non-affected herds. However, all different alleles of *aprV5* and *aprB5* encode the same amino acid sequences, indicating the existence of a single protease isoenzyme 5 in both benign and virulent strains. The genes of the basic proteases BprV and BprB also exist as various alleles. However, differences found in samples from affected *versus* non-affected herds do not reflect the currently known epitopes that are attributed to differences in biochemical activity. The data of the study confirm the prominent role of AprV2 in the virulence of *D. nodosus* and shed a new light on the presence of the other protease genes and their allelic variants in clinical samples.

1. Introduction

Ovine footrot is a highly infectious disease caused by the Gram-negative bacterium *Dichelobacter nodosus* (formerly known as *Bacteroides nodosus*). It is present in many countries and has recently been reported in Europe (Moore et al., 2005; Belloy et al., 2007; Zhou et al., 2010; König et al., 2011; Frosth et al., 2012; Gilhuus et al., 2013). This debilitating disease is considered to be one of the most important causes of lameness in sheep flocks (Wassink et al., 2003; Abbott and Lewis, 2005). Apart from the animal ethics issues evoked by the painful condition, the lameness is of considerable economic importance in the alpine area. Here, foraging involves major walking distances. As a consequence, the disease is responsible for losses in meat, wool

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and milk production, and it increases labour and management efforts relating to treatment and eradication (Nieuwhof and Bishop, 2005; Wani and Samanta, 2006; Green and George, 2008).

D. nodosus colonises the damaged interdigital skin and is found in large quantities in the superficial layers of the early footrot lesion (Egerton et al., 1969; Calvo-Bado et al., 2011; Witcomb, 2012). Macroscopically, the condition is characterised by necrotising inflammation of the interdigitial skin; a pasty foul smelling scum accumulates and necrotic separation of the horn wall from underlying tissue occurs (Green and George, 2008). Clinical presentations vary and are classified with different scoring systems (Stewart and Claxton, 1993; Conington et al., 2008). Although the disease has a wide spectrum of severity, generally three forms are recognised; benign, intermediate and virulent footrot (Stewart and Claxton, 1993). Farming management and favourable environmental factors influence the spread and progression of the disease (Depiazzi et al., 1998; Wassink et al., 2003). However, it is the nature of the causative bacterial strain, which is decisive for the initiation, and potential severity of an outbreak (Whittington, 1995; Kennan et al., 2010, 2011). A number of virulence factors have been identified in *D. nodosus*, as *e.g.* the virulence associated gene regions *vap* and *vrl* that are preferentially present in virulent strains and may therefore be indicators of virulence (Katz et al., 1991; Billington et al., 1996). Type IV fimbriae and extracellular proteases are essential for virulence of *D. nodosus* (Kennan et al., 2001, 2010). Fimbrial surface structures act as a bacterial antigen with corresponding classification into 10 serogroups (Claxton et al., 1983; Chetwin et al., 1991). Moreover, type IV fimbriae are virulence factors essential for twitching motility and cell adherence (Han et al., 2008) but also they are directly related to protease secretion *via* a type II secretion-like pathway that utilises the type IV fimbrial apparatus (Han et al., 2007).

Extracellular subtilisin-like serine proteases (or subtilases) are commonly produced as pre-pro-precursors in a wide variety of organisms such as bacteria, archaea, fungi and eukaryotes. They are activated extracellularly by cleavage of the non-catalytic N-terminal pre-pro region and the C-terminal domain. Most of them have a broad substrate specificity and are required for either defence or growth on protein-containing substrates (Siezen and Leunissen, 1997). This protein digesting process, as a source of amino acids and energy precursors, has also been postulated for *D. nodosus* (Myers et al., 2007). More importantly, however, the ability to produce subtilases is a key virulence factor in *D. nodosus* (Kennan et al., 2010; Wong et al., 2011; Han et al., 2012). Isolates are currently routinely distinguished by the elastase test first developed by (Stewart, 1979) and by the gelatine-gel test (Palmer, 1993); they measure quantitative elastase activity and protease thermostability, respectively. Virulent strains produce the more heat stable acidic proteases AprV2 and AprV5 and the basic protease BprV; more benign strains produce the less thermostable enzymes AprB2, AprB5 and BprB. AprV2 is essential for virulence as confirmed recently by construction of isogenic protease mutants of the virulent strain VCS1703A (Kennan et al., 2010).

Analysis of the mutants' phenotypic characteristics *in vitro* and in a pen based trial has confirmed that AprV2 is the major thermostable protease and is responsible for the overall elastase activity of virulent *D. nodosus* strains (Kennan et al., 2010). The mature virulent AprV2 protease differs from the benign AprB2 only by a single amino acid change (Tyr92Arg) (Riffkin et al., 1995) which results in important changes in the three-dimensional structure (Kennan et al., 2010). AprV5 is required for the activation both of itself and of the other two proteases (Han et al., 2012). BprV is significantly more efficient in degrading extracellular matrix components of the hoof horn than the benign BprB (Wong et al., 2011). The three proteases act synergistically (Kennan et al., 2010). Since the 1.4 Mb genome of *D. nodosus* has been fully sequenced (Myers et al., 2007) continuous research on virulence factors, invasion strategies and immunogenic potential has rapidly improved our understanding of the footrot pathogenesis. The introduction of PCR-based approaches has further improved the diagnosis of the disease; yet, the knowledge regarding epidemiology and characteristics of *D. nodosus* strains circulating in the sheep population is, at least in continental Europe, still somewhat limited.

In the present study we have analysed the alleles of the genes of the major extracellular virulent proteases AprV2, AprV5, BprV, and benign proteases AprB2, AprB5, and BprB of *D. nodosus* both from healthy and from footrot-affected sheep flocks in Switzerland, France, Germany and Norway.

2. Materials and methods

2.1. Origin of samples and strains

Within the framework of an epidemiological study on footrot a total of 1715 swab samples were collected from European sheep flocks, predominantly in 2011/2012. In Switzerland, sampling was carried out on 18 different farms. Of these, nine farms (1130 swab samples) were affected with footrot at the time of sampling, with at least one animal with clinical symptoms (a); nine other farms (248 swab samples) were not affected having had no animals with symptoms for at least two years (*n*) (Supplementary Table S1). In France, 17 farms were sampled in 2012. Thereof, nine farms (79 samples) were affected and seven farms were not affected (104 samples). One farm (farm H; 12 samples) was reported to be free from footrot but individuals were affected by foot abscess and hence the status was recorded as unclear (*u*) (Supplementary Table S1). In Germany, three farms were sampled in between 2007 and 2013. Thereof, one farm was not affected (10 samples) and two (131 samples) were affected by footrot at the time of sampling. Purified DNA from four Norwegian *D. nodosus* field isolates categorised as benign by the gelatine gel test (Palmer, 1993) with modifications (Moore et al., 2005; Belloy et al., 2007; Zhou et al., 2010; König et al., 2011; Frosth et al., 2012; Gilhuus et al., 2013) and purified DNA of *D. nodosus* type strain ATCC25549^T as a control for the virulent genotype were included in the study.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2013.11.013.

2.2. Sampling procedure

According to a scoring system adapted from (Stewart and Claxton, 1993), feet were rated as either clinically healthy (score 0) or as infected (scores 1–5). Scores per sheep were determined by the highest rated foot and this foot was sampled for subsequent analysis. Specimens were taken by cotton swabs (2 mm × 15 cm, Paul Hartmann AG, Heidenheim, Germany) from the interdigital skin and, if present, from the outer rim of a lesion. Swabs were immediately soaked for approximately one minute in 1 ml SV-lysis buffer (4 M guanindinethiocyanate, 0.01 M Tris–HCl, 1% β-mercaptoethanol) and then discarded. No special cooling was applied in the field or during the transport to the laboratory of lysed samples. Samples from France and Germany were sent to our laboratory either by courier or mail within 4–10 days. At the laboratory all samples were kept at 4 °C for up to 14 days before further handling. Samples from Germany (farm S) were obtained as extracted DNA.

2.3. DNA extraction

DNA extraction followed the protocol previously described for *Brachyspira pilosicoli* (Bürki et al., 2011) and validated for *D. nodosus* as follows: After centrifugation (4500 × *g* for 10 min) of the sample in SV-lysis buffer to remove solid debris, 30 μl of magnetic bead solution (Magnesil® RED, Promega, Dübendorf, Switzerland) were added to 500 μl of the supernatant. The mixture was incubated on a shaker for 10 min at room temperature (450 rpm). With the aid of a magnetic separator (scil® Magnetic separator 24, Promega) the DNA bound to the beads was separated, washed once with SV-lysis buffer and twice with ethanol. DNA was left to dry for approximately 40 min and then dissolved in 50 μl of nuclease-free water. Finally, again using the magnetic rack to retain the magnetic beads, approximately 42 μl of extracted DNA eluate was retrieved and stored at −20 °C until further use.

2.4. Conventional PCR and sequence analysis

Detection of *D. nodosus* in all clinical samples was done by an in-house real-time PCR amplifying the polynucleotide phosphorylase gene *pnpA* that is specific for the species *D. nodosus* and present in all of the strains (Whittle et al., 1999) using primers TMDNpnpA-F (GCC ACA ATT TCT TCG ATT AAA CG), TMDNpnpA-R (AAT TGC CGC AAC TGA TAA AGC) and the probe TMDNpnpA-P (CGT GCA TTC GCC GCG GC). A selection of 75 samples from affected and non-affected farms in Switzerland, France and Germany were chosen, based on their low Ct values, for further analysis (Supplementary Table S1). In these samples the genes encoding for extracellular serine proteases *aprV2* (acidic protease virulent isoenzyme 2), *aprB2* (acidic protease benign isoenzyme 2), *aprV5* (acidic protease virulent isoenzyme 5), *aprB5* (acidic protease benign isoenzyme 5), *bprV* (basic protease virulent) and *bprB* (basic protease benign) were amplified by PCR using primers listed in Table 1. Each 30 μl reaction mixture contained 1 × reaction buffer, 5 mM $MgCl_2$, 1.2 pmol of each primer, 0.25 mM of each dNTP (Roche, Rotkreuz, Switzerland), 2.5 U FirePol DNA Polymerase (Solis BioDyne, Tartu, Estonia), and 2.5 μl of DNA lysate as a template. After initial denaturation at 94 °C for 3 min, the mixture was subjected to 35 amplification cycles with denaturation at 94 °C for 30 s, primer annealing at 58 °C (55 °C for *aprV5/B5* and *bprV/B*) for 30 s and elongation at 72 °C for 2 min. There was a final extension step at 72 °C for 7 min. Amplification products were analysed by gel electrophoresis. If no PCR product was visible on gel, 1 μl of the amplification was used as a template in an identical reaction mixture of a second step PCR. The amplicon of the second PCR was again visualised on agarose gel. PCR products were purified with the High Pure PCR Product Purification Kit® (Roche) for DNA sequence analysis using the Big Dye Terminator Cycle Sequencing Kit® (Applied Biosystems) with the primers listed in Table 1. The sequencing products were purified by ethanol precipitation and run on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems).

For each allele of *aprV2* and *aprB2*, a representative sequence of the 436 bp fragment was submitted to the GenBank database: ATCC25549^T, accession number

Table 1
Sequences and positions of primers and probes used for PCR and DNA sequencing.

Target gene	Primer/probe name	Sequence (5′→3′)	Position[a]	Amplicon size
aprV2/B2	Dnod_Apr-L	TCC AAC CGC TGC TCC AAA TG	381–400	436 bp
aprV2/B2	Dnod_Apr-R	ACC ACC GCA ACG ACC CAA TG	797–816	436 bp
aprV5/B5	APRV5_seq1	CTG TTG CCG TAG TAG ATA CAG	497–517	1107 bp
aprV5/B5	APRV5_seq5	TTA ATT GAC TCG GAT CAG TAT G	1768–1788	1107 bp
aprV5/B5	APRV5_seq2	CAA CCA TTA TCG TTG CAG	992–1012	
aprV5/B5	APRV5_seq4	CTG CTG CAA CGA TAA TGG TTG	992–1012	
bprV/B	Dnod_Bpr-L	CGT GGA TAC TGG AAT TTT GG	537–556	1256 bp
bprV/B	Dnod_Bpr-R	GCG CTT TAT CGG TTA CTT TC	1773–1792	1256 bp
bprV/B	Dnod_Bpr-2	CTT GTC CAA CGT CAA TAG TTG	1187–1207	
bprV/B	Dnod_Bpr-3	TCG CTT AAT TCT GAC GGT G	1325–1345	
bprV/B	Dnod_Bpr-4	TGC CGC GGT AAC CAA TAA CG	759–778	
bprV/B	Dnod_Bpr-6	GTT GCC ATT CGC GAT TTA AG	1525–1544	

[a] Positions correspond to nucleotide sequences of: *pnpA* of *D. nodosus* benign strain C305 (accession number EU074163), *aprV2* of *D. nodosus* virulent strain A198 (accession number L38395), *aprB2* of *D. nodosus* benign strain C305 (accession number FN674446), *aprV5* of *D. nodosus* genomeVCS1703A, gene ID 5122149 (accession number NC009446), *bprV* of *D. nodosus* genome VCS1703A (accession number CP000513) and *bprB* of *D. nodosus* benign strain 305 (accession number L37754).

KF452301; Sample 1106, accession number KF452302; Nor2C, accession number KF452303; 3NA61, accession number KF452304; 3NA11, accession number KF452305; and Nor4B, accession number KF452306. Sequence data from all *aprV5/aprB5* alleles (accession numbers KF452307–KF452313) and *bprV/bprB* alleles (accession numbers KF452314–KF452321) were deposited at the GenBank database.

2.5. Data analysis and bioinformatics

Nucleotide sequences were assembled and edited using Sequencher® v.4.0.5 software (GeneCodes, Ann Arbour, MI). Comparison and pairwise alignments of the edited sequences were done using GeneDoc v.2.6.002 software. All nucleotide sequences were separately aligned with their reference gene sequences obtained from GenBank. An average linkages tree was derived using the unweighted-pair group method (UPGMA) in MEGA v.5.1 software. Identity analyses were carried out using BLAST (http://www.ncbi.nlm.nih.gov/). Note that, as a convention in this work, we use positions of nucleotides (nt) of the various genes starting with the start codon of the pre-protein. For amino acid (aa) positions we use the numbering starting at the first aa from the mature protein sequence.

3. Results

3.1. Detection of D. nodosus positive clinical material

In order to define *D. nodosus* positive samples for further analyses of protease genes, 1715 samples from

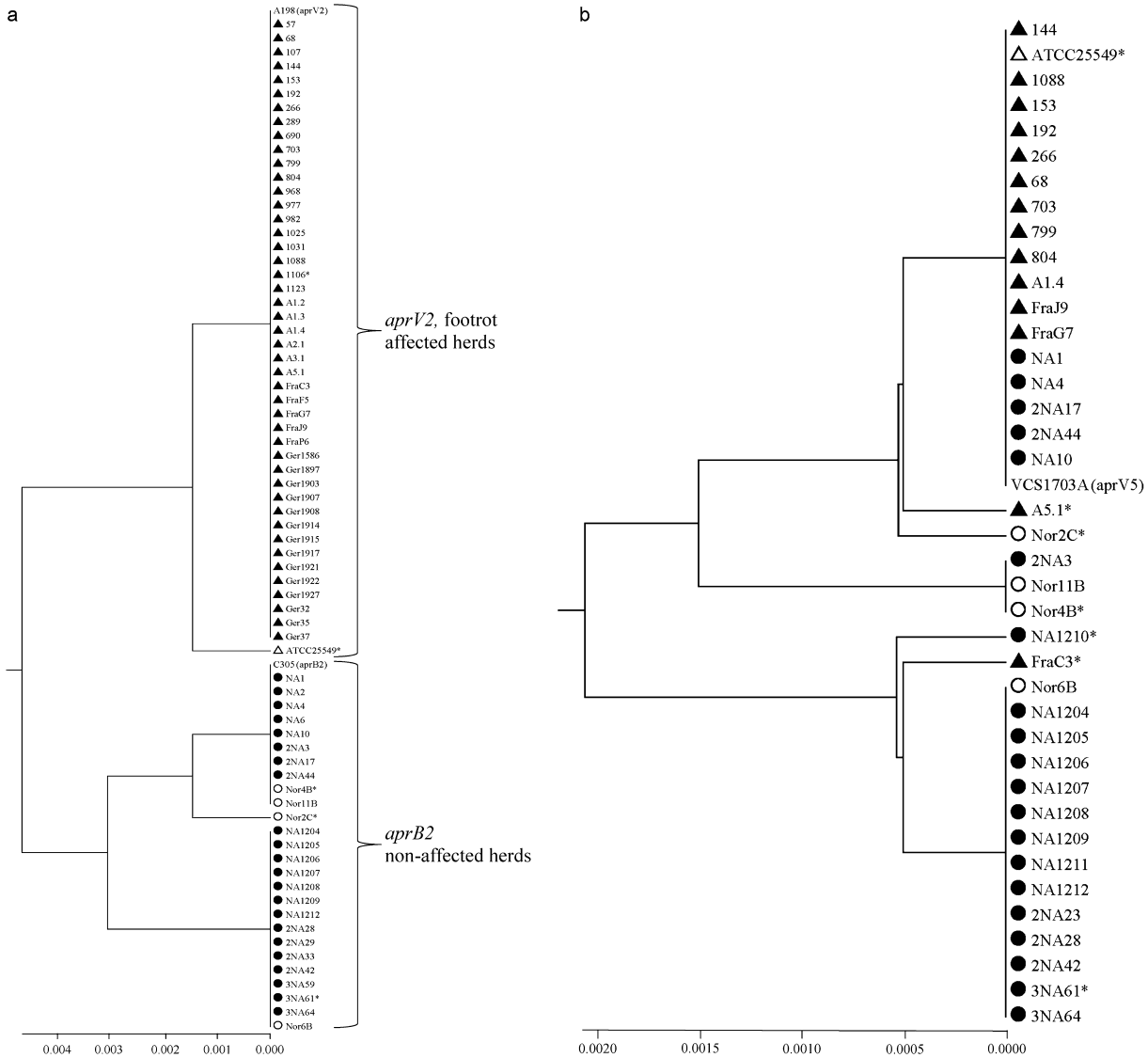

Fig. 1. UPGMA trees for *aprV2/B2* (a), *aprV5/B5* (b), and *bprV/B* (c). Footrot status of the sampled farms is indicated by a triangle (affected) and a circle (non-affected) to the left of the sample name. The benign strains from Norway as well as the virulent type strain ATCC25549^T are labelled with non-filled shapes. Reference sequences of the genes taken from GenBank are indicated by strain number but without label. Samples of which the sequences were submitted to GenBank are labelled with an asterisk. The scale at the bottom of the figure represents genetic distance.

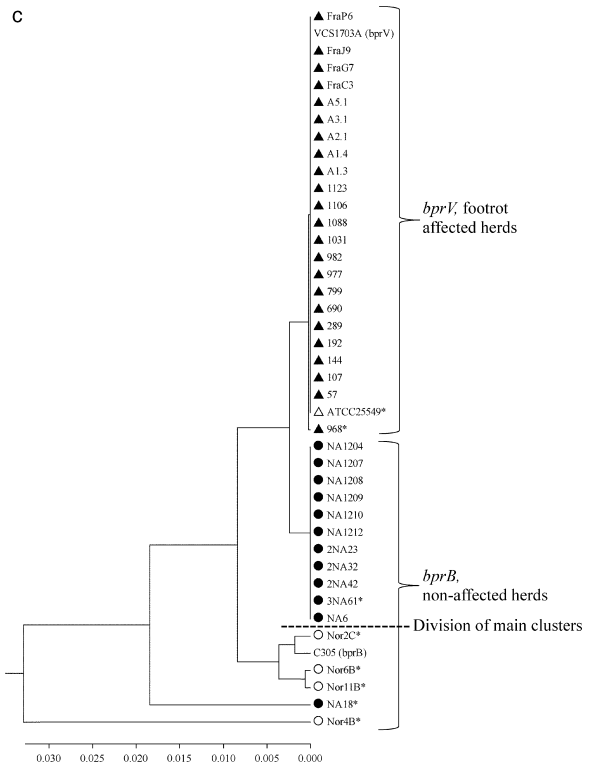

Fig. 1. (*Continued*).

clinical material were analysed using a *pnpA* real-time PCR assay. Thereof 75 samples with low Ct-values indicating high concentrations of *D. nodosus* genetic material were selected for further analysis. Of these samples 44 originated from clinically affected farms, 30 samples from non-affected farms and one from a farm with unclear status (Supplementary Table S1).

3.2. Amplification and sequencing aprV2 and aprB2

Amplification and sequencing of the 436 base pairs (bp) fragment from *aprV2* was successful in all 45 samples from affected farms and in the virulent type strain ATCC25549^T. All of them showed identical sequence compared to the reference nucleotide (nt) sequence of virulent strain A198 (accession no. L38395). All of them contained the virulent specific TA nucleotides at positions 661/662 resulting in a tyrosine residue at amino acid 92, which is required for the activity of AprV2 and characteristic for virulent strains (Kennan et al., 2010). As an exception, *D. nodosus* type strain ATCC25549^T showed an additional difference with a single nt change at position 547 (G547A) representing, however, a silent mutation (Fig. 1a).

Sequence analysis of the 436 bp fragment of *aprB2* was successful in 24 samples from sheep of non-affected farms, as well as the four benign Norwegian strains. They contained the CG nucleotides at positions 661/662, resulting in an arginine residue at amino acid 92, which is characteristic for the thermolabile AprB2 protease in benign strains. Additional variations were found at nt positions 738 and 744, exclusively in samples from non-affected farms and in a benign strain from Norway. Here, both C were substituted with T at these positions. Interestingly, these changes were conserved within farms; every sample from farms 9, 11 and 15 showed TAATAAT, whereas all of the samples from farm 8 contained CAATAAC. Two samples from farm 10 were recorded as YAATAAY; they showed superimposed double peaks (C/T), suggesting that both alleles were present in individual animals from this farm. Among the Norwegian isolates, one contained TAATAAT, the three others CAATAAC. Furthermore, an additional difference was observed in strain Nor2C (T600C) (Fig. 1a). However, all these additional changes are silent mutations.

3.3. Amplification and sequencing of aprV5 and aprB5

Amplification and sequencing of the 1292 bp fragment of the *aprV5* gene was successful in 11/45 samples from clinically affected farms and of the analogous *aprB5* in 20/30 samples from non-affected farms. Sequencing was done with two additional internal sequencing primers (Table 1). Both cohorts of samples showed considerable genotypic variability on the nucleotide level. When aligned to the sequence of *aprV5* of the virulent *D. nodosus* strain VCS1703A (accession number NC_009446), the alignment revealed 16 positions with substitutions of either C or T. Additionally three positions revealed the following differences: NA1210 showed a single transition (A927G) compared to all other samples. A5.1 showed a single transition (G1318A) compared to all other samples and a T at position 1041 where all the other samples showed either G or A. However, none of these nucleotide changes resulted in an amino-acid change. The DNA sequences of all samples analysed resulted in an identical amino acid sequence, independently of whether they originated from sheep with or without footrot lesions, from the virulent type strain ATCC 25549^T or from the four benign strains from Norway, and corresponded to the aa sequence derived from *aprV5* of strain VCS1703A. Fig. 1b illustrates how sequences of *aprV5* and *aprB5* are scattered among samples from sheep with footrot and from sheep of healthy herds in contrast to *aprV2* and *aprB2* (Fig. 1a), where *aprV2* correlates with diseased sheep and *aprB2* with sheep from symptom free herds.

3.4. Amplification and sequencing of bprV and bprB

Amplification and sequencing of the 1256 bp fragment of *bprV* was successful in 24/45 samples from affected farms and of *bprB* in 18/30 samples from non-affected farms. In total, 46 bp differences between the *bprB* reference sequence of benign strain 305 (accession number L37754) and the *bprV* reference sequence of the virulent strain VCS1703A (accession number CP000513) were evidenced in the alignment; they resulted in 12 amino acid changes between the two.

All of the samples from affected farms showed an identical nt sequence to gene *bprV*, with the exception of sample 968 that has a G at nt 1453, which, however, does not affect the amino acid sequence.

The *bprB* sequences of samples from unaffected farms showed many more differences resulting in 22 amino acid changes (Fig. 1c). Among them, amino acid positions 180 and 182 are of particular interest as changes at these positions affect the backbone of the protein structure (Wong et al., 2011). At these positions all virulent samples as well as, surprisingly, all samples from non-affected farms from Switzerland revealed glycine residues corresponding to the virulent BprV. Only the 4 benign Norwegian strains showed aspartic acid residues corresponding to the benign BprB. The UPGMA tree (Fig. 1c) shows the variability of *bprB* in benign samples in contrast to the constant *bprV* in samples from footrot-affected sheep.

Two amino acid positions consistently correlated with the farm status from which the samples were derived; 66Asp and 100Lys, corresponding to BprV, were only present in samples from sheep with footrot; and 66Lys and 100Arg, corresponding to BprB, were only present in samples from healthy sheep. NA18 was an exception (unaffected animal) that showed the greatest differences to any other sample and is also different at position 100 (see also Fig. 1c).

4. Discussion

Subtilisin-like extracellular proteases are essential virulence factors in *D. nodosus*. This anaerobe only has a reduced capacity to synthesise amino acids and hence appears to be dependent on these enzymes to degrade proteinaceous substrates of the host's extracellular matrix for its supply of amino acids. The genetic and phenotypic characteristics of the three key enzymes and their role in the pathogenesis of footrot have been investigated extensively in virulent and benign *D. nodosus* reference strains (Kennan et al., 2010; Wong et al., 2011; Han et al., 2012). They comprise two acidic proteases AprV2 and AprV5 and one basic protease BprV in the virulent type of *D. nodosus* and the three analogous proteases AprB2, AprB5 and BprB in the benign *D. nodosus*.

The acidic protease AprV2 was shown to play a determinative role in virulence as an *aprV2*$^{-}$ mutant of a virulent *D. nodosus* strain resulted in an attenuated strain that could be reverted to virulence by *trans*-complementation with the *aprV2* gene (Kennan et al., 2010). The difference of enzymatic characteristics between AprV2 and AprB2 was shown to be due to a single aa change at position 92, due to two nt mutations (TA/GC) in the gene. Our molecular epidemiological approach using subtilase-based virulotyping fully confirms this result in *D. nodosus* samples from sheep flocks in Europe. All of the samples from flocks with affected individuals revealed nucleotides TA at positions 661/662, resulting in Tyr92 in AprV2, whereas all of the samples from sheep flocks with no signs of footrot as well as the Norwegian benign *D. nodosus* strains showed GC at these positions, resulting in Arg92 of AprB. All other differences were exclusively found in benign samples showing that *aprB2* allows more variability than *aprV2*. Furthermore, the differences in the various alleles of *aprB2* are silent mutations that would only have a minor potential effect on the phenotype by means of codon usage frequency. However, it has to be noted that the various alleles found in *aprB2* were farm specific.

The acidic protease AprV5 has recently been shown to be essential for self-processing and for optimal processing the other two proteases *via* its C-terminal domain. The deletion of the latter has led to a delayed expression of extracellular protease activity (Han et al., 2012). Our DNA sequence analysis revealed three different alleles of *aprV5* (from affected herds) and five different alleles of *aprB5* (from non-affected herds). None of the nucleotide differences, however, affect the amino acid sequence and hence they have either only a minor effect on the phenotype or none at all. It is important to notice that the various alleles are not specific to virulent or benign samples of *D. nodosus*, but are distributed across samples from diseased and from non-diseased sheep (see Fig. 1b). Hence we could only evidence one phenotype of the acid protease isoenzyme 5 in both virulent and non-virulent *D. nodosus*, which would make a unique designation as Apr5 most appropriate. This unique phenotype may be explained by the hypothesis that the acid protease isoenzyme 5 possesses a core function in the processing of AprV2 and BprV (Han et al., 2012) as well as of AprB2 and BprB.

The basic protease BprV has been shown to act synergistically with the two acidic proteases AprV2 and AprV5 (Kennan et al., 2010). The catalytic domain of the virulent type, BprV, shows 96% amino acid identity with the benign type, BprB (Lilley et al., 1995). Recently it was shown that both of the two basic enzymes preferentially target ovine keratin; and more importantly, that BprV degrades this substrate more efficiently than BprB. Structural and functional comparisons of BprV and BprB revealed that the two glycine residues at positions 180 and 182 are involved in the enzymatically important S1 pocket (Wong et al., 2011). Commutations of these residues by aspartic acid as found in the benign BprB play a key role in the difference of elastase activity between the two types of proteases. This was evidenced *in vitro* by substitutions Asp180Gly and Asp182Gly of BprB, which resulted in an increase of the enzymatic activity to a level similar to BprV (Wong et al., 2011). Our sequence analysis of BprV from samples of animals with footrot and of BprB from samples of healthy sheep revealed several interesting features. First, the samples cluster in two main groups, one relatively tight cluster with all samples from France, Germany and Switzerland, except for one, together with the type strain and the reference sequence for BprV (Fig. 1c). In this cluster all samples contain characteristic glycine at residues 180 and 182, independently of whether they originate from affected or non-affected farms. This main cluster is then subdivided into two subclusters, one containing the virulent samples including the virulent type strain ATCC25549^{T} and the reference sequence for BprV and the second subcluster containing samples from sheep of non-affected herds. These two subclusters differ in aa 66 and 100, although these sites have not been identified to be involved in differences of protease activity. From these data we speculate that, either the asparagine at position 66 and the lysine at position 100 also contribute to the functional stability of BprV, or that the basic proteases BprV and BprB only play a minor role in the virulence of *D.*

nodosus. The second main cluster only contains *bprB* sequences from the four benign isolates from Norway and from one benign sample from Switzerland as well as the *bprB* gene of the benign reference strain (Fig. 1c). All these alleles, although they strongly differ from each other, reveal the aspartic acid signature of the benign BprB at aa 180 and 182.

Taken together, our molecular genetic epidemiological data based on the three subtilisin-like extracellular proteases of *D. nodosus* show that the acidic proteases AprV2 and AprB2 clearly correlate with both the virulence trait of the respective *D. nodosus* and the clinical status of the herd from which samples were taken. We interpret the presence of virulent *D. nodosus*, based on the presence of *aprV2* in some individuals without any symptoms, to be due either to early infection or to clinically unapparent carrier cases. Although our genetic approach does not distinguish between live bacteria and cell debris containing bacterial DNA the study revealed a relatively high proportion of sheep with footrot or lameness concomitant with the presence of virulent *D. nodosus*. The data from our genetic study will be fundamental for the design of novel diagnostic tools for assessment of footrot epidemiology.

5. Conclusions

The virulotyping approach presented here may well further advance the knowledge on molecular epidemiology of *D. nodosus* in sheep populations in continental Europe. Our study reveals the alleles of three major protease genes of *D. nodosus*. Extracellular subtilases are essential enzymes in the pathogenesis of footrot. They are involved in the characteristic tissue destructive features of the disease. We used clinical material from sheep either suffering from or in contact with footrot, and clinical material and *D. nodosus* isolates from sheep of disease-free flocks originating from four European countries. By virulotyping based on the gene sequences of *aprV2*, *aprV5*, *bprV*, *aprB2*, *aprB5 and bprB* we found that *aprV2* is the most reliable indicator for virulence. Molecular genetic analysis of the *aprV2* and *aprB2* gene sequences substantiate the prominent role of the allelic differences TA/CG at nt 661/662; the corresponding aa change Tyr92Arg distinguishes between the thermostable protease AprV2 in virulent and the thermolabile AprB2 in benign *D. nodosus* (Kennan et al., 2010). Although for the genes *aprV5* and *aprB5* several alleles were found in virulent and benign samples, none of the differences affect the aa sequence of the protein, resulting in the same protease isoenzyme 5 in both virulent and benign *D. nodosus*. The basic protease genes *bprV* and *bprB* exist as various alleles that affect the aa sequence. However, the differentiation between *bprV* from virulent and *bprB* from benign *D. nodosus* involves aa other than those known from structural protein analyses.

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